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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) We hypothesize that chromosomal loss of the Y chromosome in prostate cancer is a specific tumorigenic event. Our studies are to prove whether loss of a specific region of the Y chromosome is indicative of a tumor suppressor gene. We transferred the Y chromosome by microcell mediated chromosome transfer into PC-3 cells to determine if it suppressed tumor growth. Of the 75 mice injected with several independent clones only one produced a tumor in nude mice in contrast to control PC-3 cells. Our second approach is to determine the frequency of loss of the Y chromosome in prostate tumors. PCR analysis of Y specific loci on prostate cancer samples and FISH is used to identify the region of loss. Smaller chromosomal fragments are being used in the transfer experiments to define the region. Data from these experiments suggest that a tumor suppressor gene is located near the selectable marker on the short arm. The second approach is to develop a genomic microarray specific for the Y chromosome. BAC clones have been isolated and arrayed. Successful identification of a gene on the Y chromosome will provide a marker that may aid in the diagnosis and prediction of prognosis of prostate cancer.				
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INTRODUCTION:

Loss of the Y chromosome has been noted in fluorescent in situ hybridization (FISH) experiments on sections of prostate tissue by other investigators [Hum. Pathol. 27:720(1996); Cancer Genet. Cytogenet. 66:93(1993); and Cancer Res. 54:4472(1994)]. However, the molecular probes used have been either Y heterochromatin or centromeric probes. Consequently, our experiments are designed to determine whether specific regions of the Y chromosome are lost in prostate tumors. Chromosomal loss is a hallmark of a tumor suppressor gene. Functional proof that a tumor suppressor gene lies on the Y chromosome comes from experiments using microcell transfer of a human Y chromosome with a selectable marker. If a tumor suppressor is located on the Y chromosome, transfer of this chromosome into a tumor cell line will result in the suppression of tumor formation in nude mice. We are determining loss of Y chromosome DNA by PCR analysis of microdissected tumor samples and also hybridization of genomic DNA arrays we have formulated for the Y chromosome. Probes to be used in in situ hybridization experiments are also being isolated to test tumor sections. These experiments are designed to determine if a tumor suppressor gene resides on the Y chromosome and to provide evidence for the location of this gene.

REPORT BODY:

1 Progress

Statement of Work

The timetable for the Statement of Work was changed and reported in the 12 month progress report.

A. Preparation of Y chromosome markers

Months 0-6.

-Develop PCR assays for Y chromosome markers

We have developed a panel of Y chromosome PCR markers that are distributed along the whole of the Y chromosome. These markers are specific for the Y chromosome and do not prime X chromosome markers or Y sequences from other species. This is especially important in assaying somatic cell hybrids. Assays have been developed for 71 markers. Most of the markers were localized on the G3 radiation hybrid map, but 15 were mapped on this panel to produce a comprehensive map of the Y chromosome (Table 1).

-Characterize clones for Y chromosome markers

To identify the correct clone to use as starting material for transferring the Y chromosome we assayed 33 clones from a rodent cell containing the Y chromosome that we had marked with a selectable marker. Eighteen clones were positive for at least one Y chromosome locus. However, no individual clone was positive for all Y chromosome markers. Consequently, it was not possible to transfer the intact Y chromosome into PC-3 cells from these input clones. We delayed the Y chromosome transfer until we determine which part of the Y chromosome is

most commonly deleted and then we could transfer the Y chromosomes containing or lacking that specific region.

B. Preparation of microcell hybrids

Months 12-18.

- Transfer the Y chromosome into PC-3 cells

pT+km3 clones 2-6B and 1-5B (rodent A9 + Y chromosome) were chosen for chromosome transfer experiments. Micronuclei were produced by prolonged exposure of the pT+km3 clones to colcemid and centrifugation through cytochalasin B. These micronuclei were fused to PC-3 prostate cancer cells using polyethylene glycol. Microcell hybrids were selected using histidinol.

- Isolate independent clones

20 independent clones were isolated from the 2-6B pT+km3 x PC-3 fusion and 18 from the 1-5B fusion. Each clone was tested for 15 markers along the Y chromosome. Clones varied in composition for positive Y chromosome markers. Five clones were chosen for injection into nude mice: some contained all the original markers while others did not. (See Table 2)

- Inject clones into nude mice

Each clone was injected into 5 nude mice at 2.5×10^6 cells per mouse. The parental PC-3 cell was used. (See Table 3). In addition to PC-3 hybrids we injected the microcell hybrids with the human Y on a mouse A9 background. A9 cells form a fibrosarcoma when injected into nude mice. To our surprise, the Y chromosome also has an inhibitory effect on the A9 cells which normally produce fast growing tumors. After 6 weeks no sign of tumor formation was present. (Figure 1)

Months 19-28 .

- Continue to produce microcell hybrids

We made additional sets of hybrids from 2-2 C pT+km3 transferred into PC-3 cells.

- Try other prostate cancer cell lines for microcell transfer

A karyotype of DU145 cells indicates that it has a Y chromosome. Analysis of 20 markers by PCR indicated that all were present.

C. PCR of dissected material

Months 6-18.

- Initiate PCR analysis of dissected material

We have developed PCR analysis for paraffin embedded, dissected tissue samples. To do this, we developed a reliable method for the isolation of DNA from these samples. Our method reliably results in DNA that can be amplified if the fragments are 200 bp or less. Our initial studies have been with 10 primers and one primer from an autosome as a control. We examined methods for both cold PCR and also radioactive PCR.

- Determine level of contamination with normal tissue that can be tolerated

To study the issue of normal cell contamination, we have diluted DNA from normal male tissue with DNA from a female. Although we can observe differences in the amounts of PCR product produced, it is still difficult to determine whether there is an increase in ploidy with loss of the Y chromosome versus just loss of the Y chromosome. It may be necessary to develop real time PCR to quantitate the differences.

- Initiate analysis of tumor tissue samples by PCR

We have initiated the analysis of tumor samples. For our initial studies, we have available to us slides from 20 normal samples, 29 from tumors only, and 7 with normal and tumor samples. Of these samples we have examined 8 primers on 13 tumor/normal pairs. Loss of the Y chromosome appears to center around a specific locus, DYS288. Dr. Troyer has amassed over 100 patient samples for us to further this study.

Months 19-28

D. Narrowing the region of deletion or tumor stimulation

Months 13-18

- Identify PAC/BAC clones corresponding to loci on the Y chromosome

Because of the advances of microarray technology and the availability to us of a DNA arrayer, we have decided to aggressively pursue this approach to DNA loss. In addition to DNA arrays, these clones will be used as hybridization probes for FISH experiments. We have identified markers that are spaced over the length of the Y chromosome as the basis for isolating BAC clones specific for the Y chromosome. Clones were identified in the RPCI-11 library by screening filters with the overgo method. This method using overlapping oligonucleotides (24 mers with an 8 bp complementary overlap) specific for each marker. The single stranded region is filled in using radioactive nucleotides. The probes are then hybridized to the filters in pools. Positive clones are decoded (the filters are stamped in duplicate with a unique pattern). Additional clones were identified from sequence analysis of the rough draft of the human genome.

- Isolate the clones, subclone and assay for markers by PCR

Primary positives from the hybridization were struck out on agar plates and two subclones were picked for each positive address. Since the markers were hybridized in three pools, each pool was tested to identify the correct positives by PCR. 195 BACs corresponding to 71 markers have been identified to date. Each of these clones originates from the Y chromosome by their marker content. As stated previously, we have tested these primers for their specificity to detect Y chromosome sequences.

- Formulate array for identifying regions of chromosomal deletion

Our initial step has been to develop 71 markers spanning the Y chromosome. These BACs encompass ~12.6 Mb of the Y chromosome. As we test tumor samples for loss of hybridization signal, we will determine which region is the highest region of loss. We will then saturate this region with more BAC clones in an effort to find the smallest overlapping region of loss.

Months 19-24

- Develop FISH of tumor tissue

- Analyze tumors with Y repetitive probes

- Analyze tumors with specific Y probes

We have analyzed prostate cell lines with a large number of markers as well as hybridized a repetitive Y probe to metaphase chromosomes. We found several deletions using these methodologies.

- Establish conditions for stamping array and hybridization

The San Antonio Cancer Center has established a DNA arraying facility. We are working closely with this facility to determine the optimum conditions for stamping the array on glass slides. We have tried a number of different methods to obtain maximal signal. We have

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also optimized methodology to amplify DNA samples using DOP PCR (degenerate oligonucleotide primer PCR).

- Establish conditions for comparative genomic hybridization using male and female DNA

Although we have tried a number of methods, the signal is not as good as it should. We continue to improve these methods.

- Assay tumor DNA samples

- Begin irradiation microcell hybrids

In examining the many microcell hybrids we have already, we find there is sufficient representation in the fragments to eliminate the need to construct irradiation microcell hybrids.

Months 25-30

- Analyze tumor hybridization to Y chromosome array

- Perform nude mouse assay on those microcell lines with varying fragments and/or BAC clones of the Y

The review of our previous progress report made the suggestion that we transfect the PC-3 cells with the histidinol marker as a control. These experiments are underway.

- Summarize all the data from the above studies.

The accomplishments we have made over the past 18 months will allow us to quickly analyze tumor samples for loss of Y chromosomal material. After a specific region is evident and this region is present in one of our donor somatic cell hybrids, we will follow through with chromosome transfer experiments. Both of these lines of evidence will either prove or refute the presence of a tumor suppressor gene on the Y chromosome.

KEY RESEARCH ACCOMPLISHMENTS:

- Introduction of the Y chromosome into PC-3 prostate cancer cells results in total suppression of tumor growth in nude mice
- Introduction of the Y chromosome into the male mouse A9 fibrosarcoma cells results in total suppression of tumor growth in nude mice
- Suppression activity is eliminated from the majority of the long arm of the Y chromosome
- A genomic array of BAC clones have been isolated for the Y chromosome
- A repository of prostate tumor samples has been established

REPORTABLE OUTCOMES:

- Abstract presented at the Society for Basic Urologic Research Fall Symposium, December, 2000
- 15 mouse somatic cell hybrids were characterized containing fragments of the human Y chromosome

- 38 microcell PC-3 hybrids identified with fragments of the Y chromosome
- Prostate tumor samples have been collected and patient data maintained in a database for >100 patients
- 195 ordered BAC clones have been identified for the length of the Y chromosome

CONCLUSIONS:

The research we have completed to date has resulted in assembling and characterizing a large number of reagents essential to test the hypothesis that there is a tumor suppressor gene on the Y chromosome. Our initial PCR analysis of prostate tumors indicates that we can detect regional loss of the Y chromosome in archival tissue. To assay a large number of markers for a large number of loci we will use genomic DNA arrays for which we have assembled the appropriate clones. Although we have just begun transfer of the Y chromosome fragments at least one region appears to stimulate tumor growth. These data have caused us to revise our hypothesis to state the Y chromosome has specific regions of loss and gain in prostate tumors that are characteristic and tumor suppressor genes and oncogenes, respectively. If there is a tumor suppressor gene on the Y chromosome, isolation of the gene will provide a means to discover the mechanisms involved in prostate tumor formation as well as suggest new treatments.

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APPENDICES:

- Table 1. PCR Markers and BAC Clones Specific for the Human Y Chromosome
- Table 2. Characterization of Somatic Cell Hybrids with Y Chromosome Markers (Y on Mouse A9 Background)
- Table 3. Summary of Deletions in the PC-3 hybrids
- Figure 1. Y Chromosome Suppresses Tumor Formation in PC-3 Cells

Table 1
PCR Markers and BAC Clones Specific for the Human Y Chromosome

Marker	G3 Dist.	BAC Clone	Marker	G3 Dist.	BAC Clone	Marker	G3 Dist.	BAC Clone
DYS253	242		DYS198	820	15H4			168C24
DYS288	372	109F19*			324D10	SHGC-1348	125	270H4
DYS260	387	109F19*	DYS289	848	55o11	DYS241	1268	174G3
DYS263	396	28F10			118L3			232G11
		46F23			8L14			71B10
		39M17			243D21	SHGC-1412	1294	
		209i22	DYS200	863	99M1	DYS206		347o22
DYS266	426	111o24			166o18	DYS214		3M3
		346G2	DYS199	887	71B21			106H16
		458F2			99M1			69C24
		140H23			166o18			59C12
		49G16	DYS201	896	462A19			23H19
		26B12	DYS213	930				56B22
		44C16	DYS211	948	20H21			26B12
D3S398	482	71A3			33H1			174G3
		161O14			211N14	DYS223		400K11
		66G17			243P9			8L14
		69A13	DYS212	966	392F24			18L5
		160E24	DYS219	1101	333E9			420P2
		71C4	DYS221	1144		DYS225		426N11
		35D7	DYS278	1153	304C24			370N2
		27E21	SHGC-7605	1199	66M18*			177i21
		116J19	DYS400	1238	1o23			400i17
DYS270	533	66M18*			160o17	DYS227		120E18
DYS271	547	44C16			137J3			126F23
DYS272	574	144J1*			109G18	DYS231		104C20
DYS277	605	119M24			270H4*			427G18
		193J24			45P11			453C1
		304C24			39P20			182H20
SHGC-9460	639	470K20			73N13			569J3
DYS279	652				55E5			489J18
DYS276	667	386L3			168C24			516L5
DYS280	704	168C24*			95B23	DYS235		71B10
DYS390	723	59K8			137A17			22E3
DYS196	760				120H6			79J10
DYS281	792	494J4			291P13			160o2
DYS243	804	494J4			86H22	DYS236		288G4

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	76N1		7C2		220o2
DYS237	71B10		492C2		137A17
	53P14	DYS392	329C15		61G1
	160o2		432A7		73N13
	86G22	DYS395	4N7		291P13
	69C24		15G18		160K17*
DYS247	288G4		516L5		492C02*
DYS258	223o4		326D9		477B5*
DYS261	500D2		250J11	DYS413	471H14
	316P19		516A11		28M17
	325D19	DYS397	8i9	DYS416	466E18
DYS378	516L5		562L14		470E22
	7C2		139M22		248H5
	489J18		8i11		175D7
	427G18		298i24		516A11
	514E2		115H13		120E18
DYS379	112N22		199M2		121N17
	427G18	DYS41	23H18	DYS417	120K3
	489J18		257D2	DYS419	372J11
	516L5		229P22		172D

Table 2
Characterization of Somatic Cell Hybrids with Y Chromosome Markers

Marker	DYS253	DYS260	DYS266	DYS272	DYS279	DYS289	DYS213	DYS212	DYS221	SHGC-7605	SHGC-14120
Cytogenetic location	p11	P11	p11	q11	q11		q11	q11	q11		
Distance in cR	242	387	426	574	652	848	930	966	1144	1199	1294
pT+km3 1-6B											
pT+km3 2-6B											
pT+km3 2-6A											
pT+km3 2-5C											
pT+km3 2-2B											
pT+km3 3-6A											
pT+km3 3-1C											
pT+km3 2-3C											
pT+km3 1-5A											
pT+km3 1-2A											
pT+km3 1-5B											
pT+km3 2-1C											
pT+km3 1-3A											
pT+km3 2-2A											
pT+km3 3-4C											
pT+km3 1-1A											
pT+km3 1-3C											
pT+km3 3-1B											
pT+km3 1-4A											
pT+km3 1-1B											

Strongly positive
Weak positive

Figure 1: Y Chromosome Suppresses Tumor Formation in PC-3 Cells

